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13. Abstract ***(Maximum 200 Words)*** *(abstract should contain no proprietary or confidential information)*
The goal of this grant was to develop effective treatments for patients with low volume metastatic disease using dendritic cell (DC)-based vaccines loaded with tumor RNA. The proposed studies were based on our previous findings that RNA loaded DC of murine and human origin are highly effective stimulators of T cells and tumor immunity. Toward this end, we have developed a robust procedure for generating immunopotent DC from breast cancer patients and methods to isolate and amplify RNA from small amounts of tumor cells were developed. Efficient loading of DC with RNA was accomplished by electroporation. To differentiate ("mature") DC we developed a novel approach whereby incubation of the DC with biological response modifiers (i.e., soluble CD40 ligand) is replaced by transfection with the corresponding mRNA. The tumor RNA loaded DC were highly effective at stimulating CTL responses in vitro. Optimized and reproducible protocols were developed for the planned clinical trials. IRB approval and IND were obtained for a phase I clinical trial in breast cancer patients. The trial has not been yet initiated due to accrual difficulties.

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A. INTRODUCTION

Whereas hormonal therapy and chemotherapy have proven somewhat effective for the prevention and treatment of metastatic breast cancer, the current limitations of systemic therapy necessitate the development of improved and/or alternative strategies. One such approach is specific active immunotherapy which is used to stimulate a tumor specific immune response capable of eliminating residual metastatic disease, and engender a state of immunity to protect the patient from recurrence of disease. Several studies have documented the exceptional ability of dendritic cells (DC) to stimulate naive T cells, both in vitro and in vivo (1, 2). Immunization using DC loaded with tumor antigens could therefore represent a potentially powerful method of inducing anti tumor immunity (3, 4). Indeed, recent studies from a number of laboratories including ours have shown that vaccination with DC pulsed with tumor antigens in the form of protein or peptide were capable of priming cytotoxic T lymphocytes (CTL) in mice and engender tumor immunity.

The major research focus of the combined laboratories of the PI and CoPI of this application was to develop effective treatments for patients with low volume metastatic disease using autologous dendritic cell-based tumor vaccines. The proposed studies were based on our discovery that RNA pulsed DC of murine and human origin are highly effective stimulators of T cells and tumor immunity. A key advantage of using tumor RNA as source of antigen is that sufficient antigen can be generated by RNA amplification techniques from small tumor specimens. Phase I clinical studies with carcinoembryonic (CEA) peptide and CEA RNA transfected DC and PSA RNA transfected DC have demonstrated (so far) the safety of this treatment (unpublished data). Furthermore, analysis of patients treated with PSA RNA transfected DC show induction of PSA-specific T cell responses in the vaccinated patients (5).

This proposal was based on the premise that activation of tumor antigen-specific T cell responses will prevent tumor recurrence and metastasis. The central hypothesis of this proposal is that vaccination with tumor RNA transfected DC against a broad repertoire of tumor antigens expressed in patients with breast cancer will constitute an effective therapy for metastatic breast cancer. Specifically, we planned to test the hypotheses that a) dendritic cell-based tumor vaccines will be effective in the treatment of metastatic breast cancer, b) immunization with a broad repertoire of tumor antigens isolated from cancer cells is superior to using defined tumor antigens, and c) loading antigens on dendritic cells in the form of tumor mRNA is highly effective and provides unique advantages over other forms of tumor antigen, namely the ability to amplify the antigenic contents of a small number of tumor cells. The primary objective of this research proposal was to develop optimal methods for using DC transfected with tumor RNA in breast cancer immunotherapy. The specific technical objectives were:

1. To optimize antigen presentation by DC transfected with tumor RNA isolated from patients with metastatic breast cancer.

2. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with RNA <u>isolated directly</u> from tumor cells.

3. To develop methods to isolate, amplify, and enrich for biologically active mRNA from breast cancer tissue.

4. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with <u>amplified</u> tumor RNA.

B. BODY-SUMMARY OF RESEARCH ACCOMPLISHMENTS

1. Optimization of antigen presentation by DC transfected with tumor RNA isolated from patients with metastatic breast cancer (Aim #1).

1-1. Optimization of RNA antigen presentation by RNA transfected DC.(SOW Yeare 1 &2)

Previous studies have suggested that RNA is taken up preferentially by immature DC , consistent with the observation that immature but not mature DC exhibit extensive phagocytic and macropinocytic activity (6). It was, however, shown that immature DC generated in the presence of GM-CSF and IL-4 are unstable and revert to monocytes when cytokines are withdrawn (7). Moreover, mature DC are more potent APC than immature DC (1, 2). We have therefore explored whether immature DC are more susceptible to RNA transfection and whether maturation of the transfected DC will enhanced their function, i.e. stimulation of CTL responses. We tested both TNF-α and CD40L previously shown to cause DC maturation in murine studies. As RNA we used CEA-specific RNA which represents a tumor associated antigen expressed in over 50% of breast cancer patients.

TNF-α. PBMC-generated DC were treated with TNF–α, before or after CEA RNA transfection. DC generated in the presence of GM-CSF and IL-4 express intermediate levels of MHC class II molecules and B7-1 (CD80) and are mostly $CD83^-$(8). Following TNF–α treatment, PBMC-derived DC "mature", which correlates with the upregulation of class II and CD80 expression and cells become $CD83^+$ (6, 8). Maturation of DC is also accompanied by an increase in antigen presentation evidenced by enhanced MLR activity of mature versus immature DC. In the experiment shown in Figure 1, DC were generated in the presence of GM-CSF and IL-4, and the large, class II intermediate immature DC (approximately 50% of the total population) were further purified by cell sorting as described previously (8).

Figure 1A shows that treatment of sorted immature DC with TNF-α causes the upregulation of CD83 expression. Figure 1B shows that "immature" DC transfected with CEA RNA and then treated with TNF-α were significantly more potent stimulators of CEA-specific CTL than DC treated with TNF-α before transfection with RNA whereas treatment with TNF-α had no significant effect on the ability of CEA peptide-pulsed DC to stimulate a CEA-specific CTL response. This experiment has been performed twice but the conditions for TNF treatment have not yet been optimzed.

In other studies ongoing in our laboratory funded by other sources, TNF treatment of murine bone marrow-derived DC also induced phenotypic maturation of the DC as evidenced by elevated expression of class II, CD80 and CD40. However, whereas the TNF treated DC were effective at presenting antigen in vitro, they were not able to stimulate CTL responses in vivo. Initial indication suggest that the TNF stimulated DC are prone to undergo apoptosis (unpublished data). We are therefore reluctant to introduce TNF treatment of DC into clinical settings as proposed in the grant application.

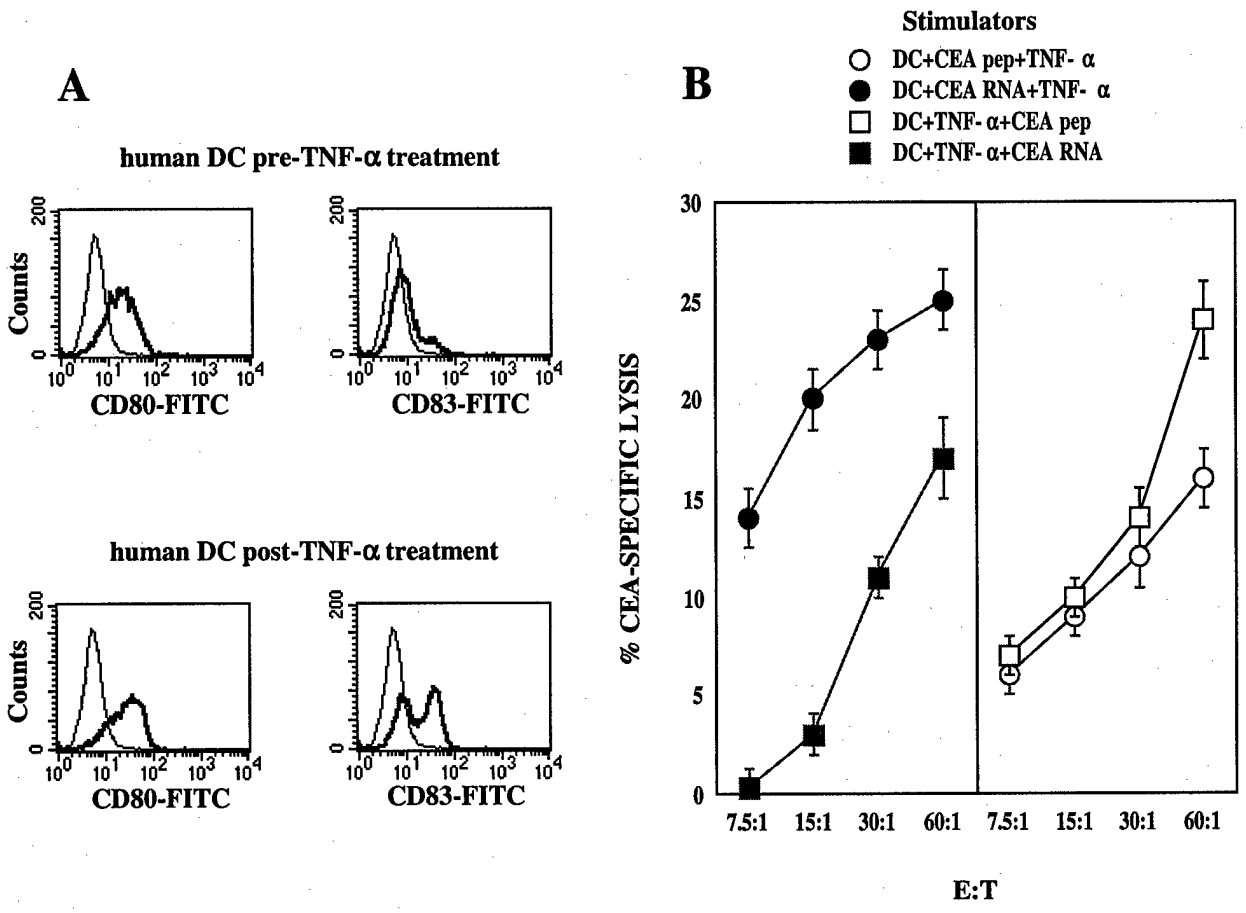

Figure 1. Effect of TNF–α treatment on the ability of CEA RNA-transfected DC to stimulate CTL *in vitro*. Panel A: DC were generated from PBMC as described (1) The large, class II intermediate cells were purified by cell sorting and analyzed pre- and post-treatment with TNF–α (100 ng/ml for 18 h) for the expression of CD80 (B7-1) and CD83. Panel B: Sorted DC were either incubated with CEA RNA or with CEA peptide followed by culture in the presence of TNF–α or cultured with TNF–α followed by RNA transfection or peptide pulsing. DC pulsed with CEA peptide and DC pulsed with HCV peptide were used as targets. Data represents the differences in the lysis of DC+CEA peptide and DC+HCV peptide.

CD40L. The effect of CD40L on RNA/transfected DC function was analyzed as described for TNF-α. CD40L was used in a soluble form as a trimer complex.CD40L is not commercially available and was obtained as a gift from Immunex corp. Figure 2 shows that treatment of DC with CD40L induces phenotypic maturation, as evidenced by the upregulation of several cell surface molecules, in particular CD80 (B7-1) and CD83.

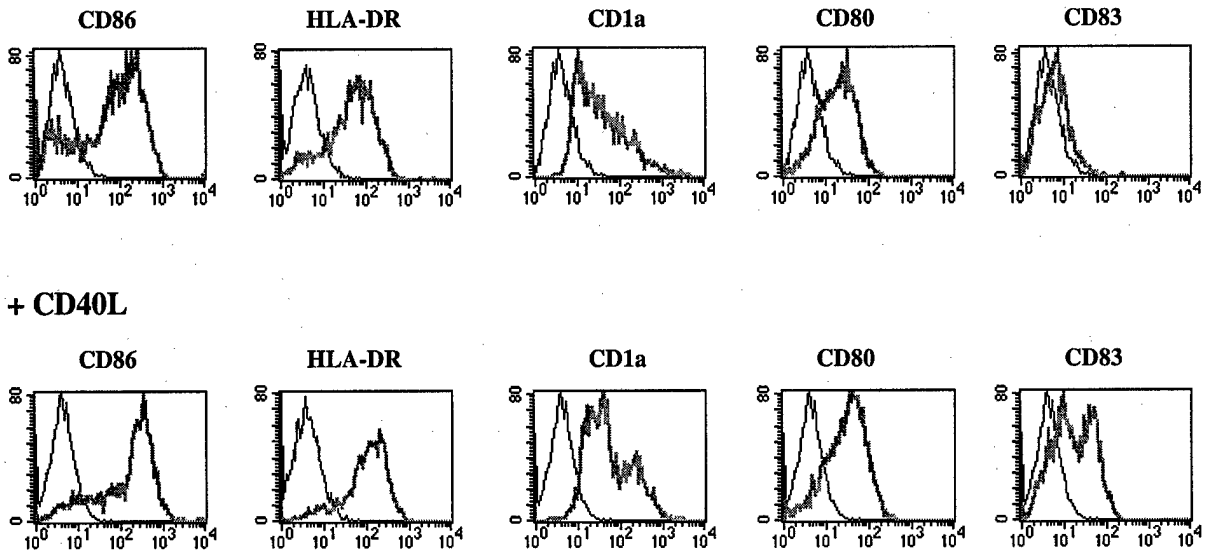

Figure 2. FACS analysis of DC before and after CD40L treatment. DC generated in GM-CSF and IL-4 for 7 days were harvested and a portion were cultured for an additional 18 h with CD40L 1 μg/ml. The DC were then stained with mAb to CD80, CD83, CD86, and HLA DR. CD83 was upregulated after CD40L exposure.

The functional consequences of CD40L treatment, i.e. stimulation of CTL responses following RNA transfection, was analyzed essentially as described for TNF-α shown in Figure 1B. The ability of DC loaded with antigen in the form of peptide to stimulate an antigen-specific CTL response was analyzed in Figure 3A. DC were pulsed with CEA peptide before or after treatment with CD40L. CD40L-matured DC were associated with greater CEA peptide-specific lytic activity if peptide pulsing was performed after maturation. Addition of the CD40L after loading peptide loading resulted in no improvement in CTL stimulation over that of DC which were not exposed to CD40L. The ability of DC loaded with antigen in the form of RNA to stimulate an antigen-specific CTL response was analyzed in Figure 3B. DC were transfected with CEA RNA pre- and post-treatment with CEA RNA. CD40L-matured DC were associated with greater CEA specific lytic activity if the CEA RNA transfection was performed before maturation. Addition of the CD40L before CEA RNA transfection resulted in similar CTL stimulation as that of DC which were not exposed to CD40L.

Thus, like in the case of TNF-α, RNA uptake was limited to immature DC. However, unlike the previous example, mature DC were more potent than immature DC in presenting peptide-antigen. The reason for this is not clear, a possible explantion is that in this instance maturation was accompanied by increases in MHC class I expression.

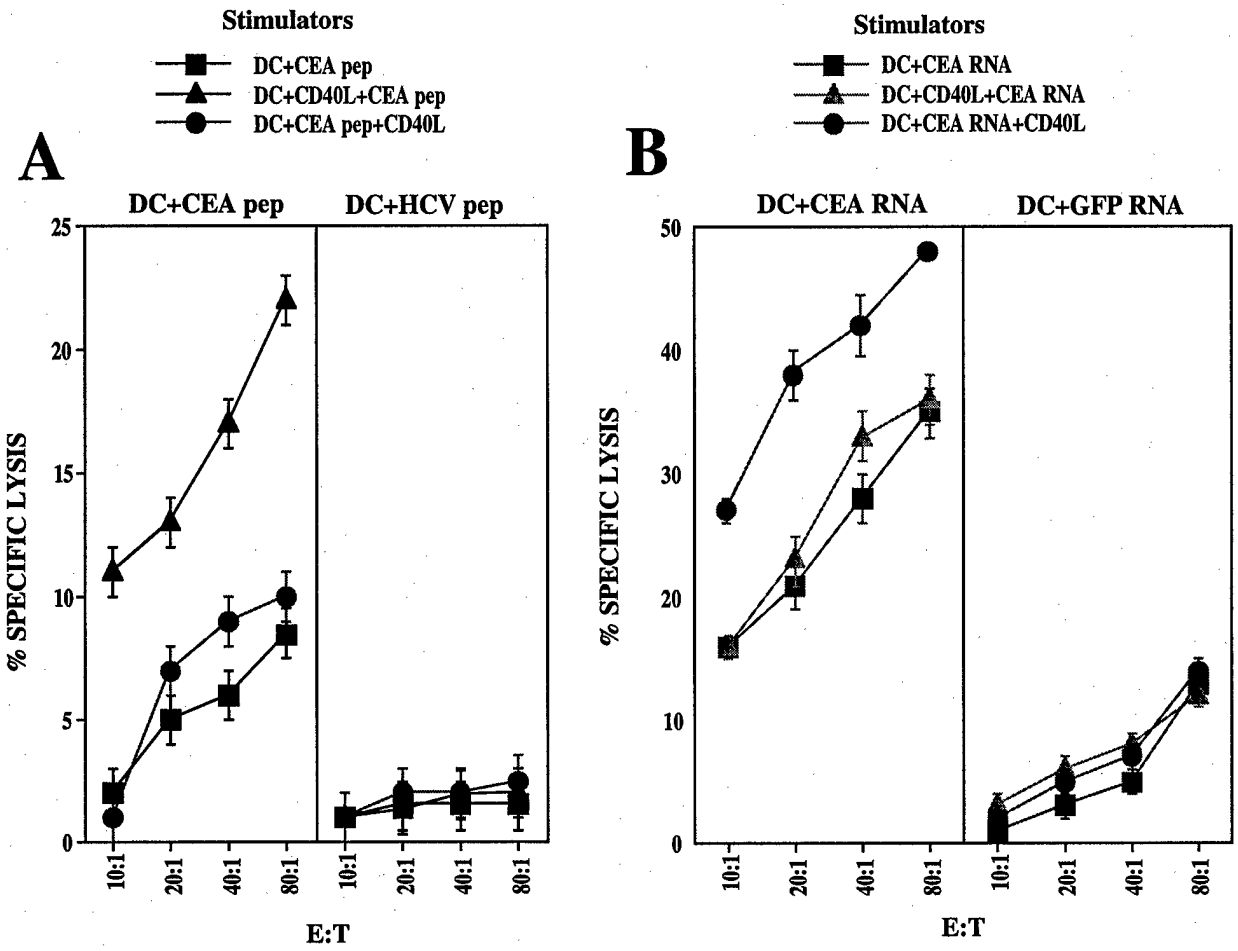

Figure 3. Stimulatory activity of DC pulsed with CEA peptide or transfected with CEA RNA pre- and post-CD40L exposure. Panel A: DC loaded with CEA peptide before and after 18 h incubation with CD40L were used as stimulators of autologous PBMC. Autologous DC loaded with CEA or HCV peptide were used as targets. Data is representative of three experiments. Panel B: DC transfected with 10 μg IVT CEA RNA before and after 18 h incubation with CD40L were used as stimulators of autologous PBMC. Autologous DC loaded with CEA or GFP RNA were used as targets.

1-2. Providing IL-4 and CD40L by mRNA transfection

Our studies summarized above have clearly suggested that loading immature DC generated I nthe presence of IL-4 and GM-CSF with RNA followed by maturation with CD40 ligand represents a potentially useful strategy to optimally load DC with antigen in a manner that maximizes the immunostimulatory function of the antigen loaded DC. However, we have encountered a major obstacle-CD40 ligand reagents, for preclinical as well as clinical studies, from Immunex Corp. was discontinued and the availability of IL-4 for DC generation became uncertain. We therefore explored a novel approach to generate and modulate DC function by replacing the use of IL-4 and CD40 ligand with the corresponding mRNAs which are transfected into the monocytes and immature DC, respectively. Use of mRNA transfection to manipulate ex vivo the function of DC offers multiple advantages over current strategies.

1. With the introduction of RNA electroporation (see below) mRNA transfection of murine or human DC is an efficient process resulting in high level of gene expression which can effect the physiology of the transfected DC in a predicted manner.

2. Generation of mRNA is a simple, rapid and cost effective protocol. The cDNAs for IL-4 and CD40L was initially isolated from PHA activated PBMC using standard RT-PCR protocols and cloned into a transcription plasmid behind a T7 promoter.The corresponding mRNA is generated in a 2 hour procedure from the cDNA plasmid using commercially available transcription kits (Ambion) (9).

3. Use of mRNA to modulate DC function offers significant advantages in clinical settings. Generation of GLP or GMP-grade mRNA and the associated regulatory approval process is a simple and cost effective process that can be readily accomplished in an academic laboratory.

4. Importantly, the use of mRNA encoded BRMs offers a broadly useful method to manipulate ex vivo the differentiation and function of cells, both for investigative studies as well as for clinical objectives.

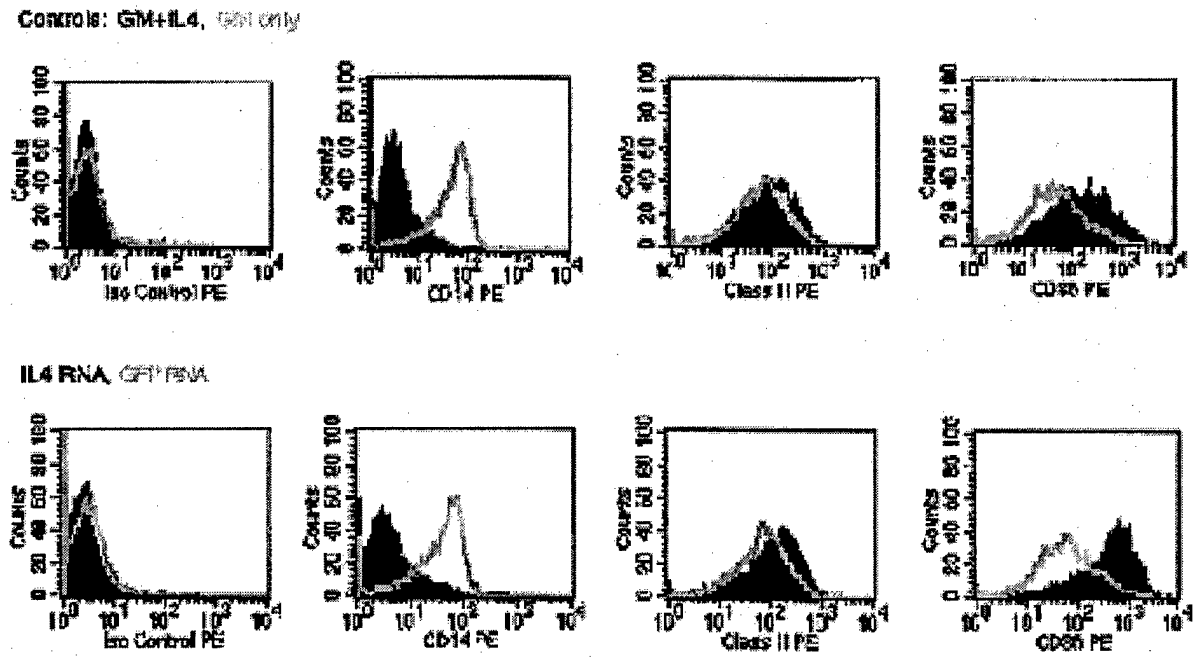

Figure 4: Generation of immature DC by transfection with IL-4 mRNA. DC were generated from monocytes in the presence of GM-CSF and IL-4 (upper pannels) or transfection with IL-4 mRNA (lower panels). DC phenotype was determined by flow cytometry as indicated.

As shown in Figure 4, for generation of immature DC, incubation of the monocytes with IL-4 can be replaced by transfection with IL-4 mRNA, thus obviating the dependence on a commercial source or gift. Likewise, DC can be matured by transfection with CD40 ligand mRNA instead of incubation with the protein reagents. Figure 5 shows that when immature DC or immature DC incubated with cytokines (CC-TNF, IL-1 and IL-6) in the absence of prostaglandin E2 (PGE) and transfected with CD40 ligand mRNA matured intto potent Th1 polarized immunostimulatory DC as jujdged by their ability to upregulate the expression of the Th1 polarizing cytokine IL-12. The CD40 L mRNA transfected DC also exhibited improved alloMLR activity (data not shown).

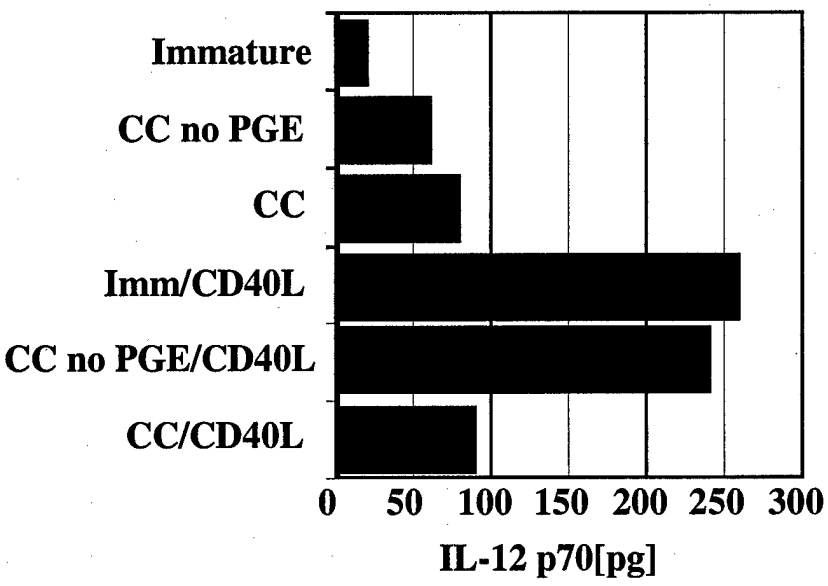

Figure 5: Maturation of human DC transfected with CD40 ligand mRNA. Human immature dendritic cells were generated from monocytes by established procedures by culturing cells in the presence of GM-CSF and IL-4. Immature DC were further incubated and the extent of maturation was assessed by measuring the secretion of IL-12 using a standard ELISA assay. DC were incubatedthe presence of a cytokine cocktail(CC) designed to induce DC maturation with or without prostaglandin E2 (PGE). When indicated, DC were also transfected with a mRNA encoding CD40 ligand (CD40L).

1-3. Transfection of DC with mRNA by electroporation.

Old and recent studies have shown that mature DC are superior stimulators of T cell responses. The DC that we have initially used-generated in the presence of GM-CSF and IL4-are immature and therefore less effective. Whereas a number of maturation protocols have been described in the literature (see also figures 1-3), in most instances the mature DC also exhibit considerable instability, namely the tendency of loosing viability. Recently, a protocol which induces maturation without loss of viability was described (7) which involves culturing the immature DC in the presence of four agents, TNF, IL-1, IL-6 and PGE2. We have adopted and optimized this maturation protocol for the human monocyte derived DC. Figure 6 shows that DC grown in the maturation cocktail acquire the mature phenotype. Not shown-the mature, but not immature, DC maintain viability for exteded period of time.

Recently, Tandeloo et al have described an electroporation-based method which results in very high levels of RNA transfer into human DC (10). In the current year we have optimized the combined RNA electroporation and DC maturation to generate superior antigen presenting cells for immunotherapy. Figure 7 shows that immature DC and mature DC cultured in the presence of the 4-reagent maturation cocktail and electroporated with GFP RNA express high levels of GFP, significantly more than generaly achieved with or without lipid.

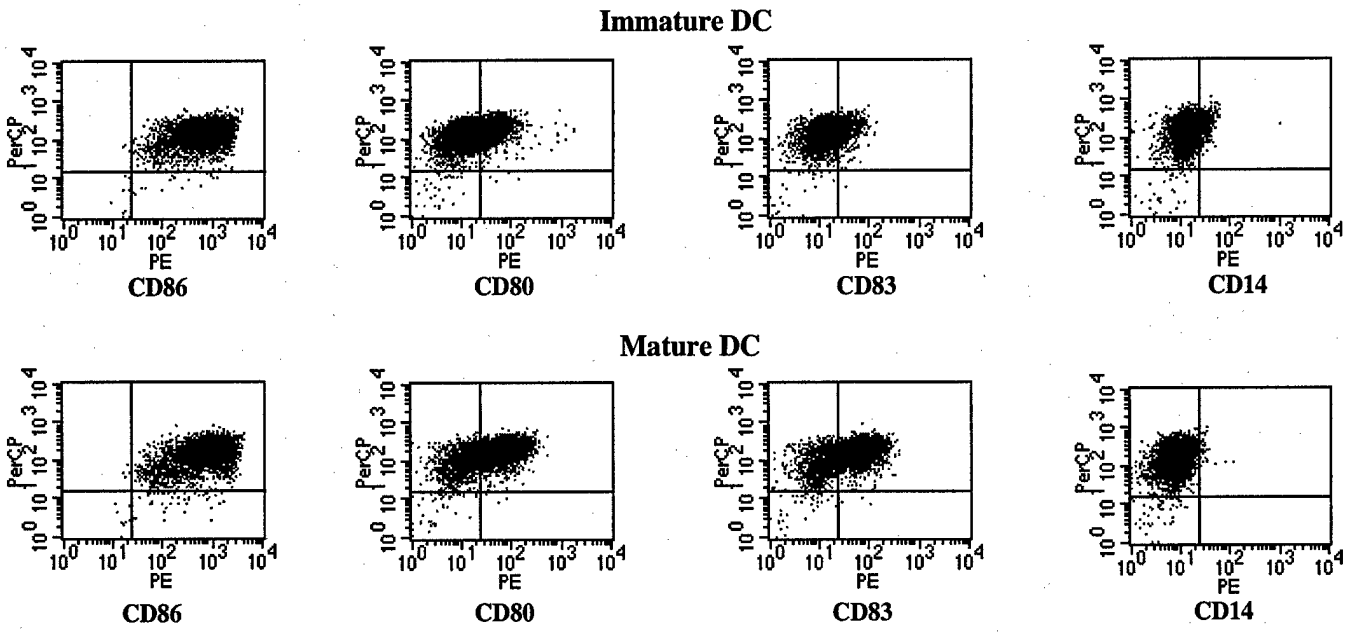

Figure 6: Flow cytometry of mature and immature DC. Immature DC were generated by culturing monocytes in the presence of GM-CSF and IL4 for 7 days. DCs were matured by 24 hour culture in the presence of IL-6, IL-1β, TNF-α and PGE_2. Immature and mature DC were immunostained with antibodies and analysed by flow cytometry. Both immature and mature DC expressed class II and B7-2 (CD86) but not CD14. Maturation was accompanied by increase in the expression of B7-1 (CD80) and CD83.

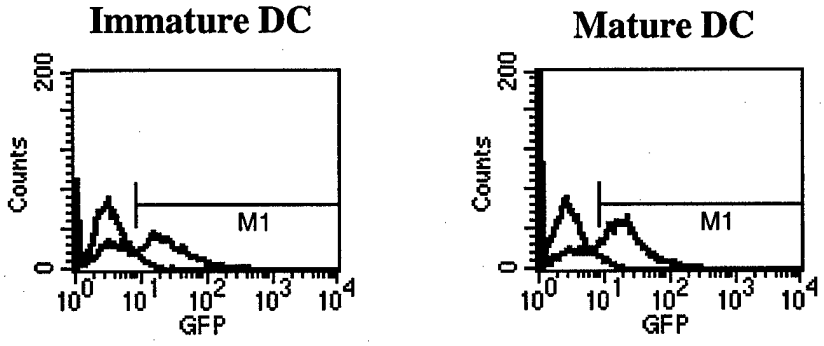

Figure 7: GFP RNA transfer into DC by electroporation. Immature and mature DC (shown in Figure1) were electroporated with RNA encoding GFP and influenza M1 (as negative control) and analyzed by flow cytometry. 60% and 75% of immature and mature DC, respectively have shown expression of GFP

Figure 8 shows that GFP mRNA electroporated DC stimulate CTL responses in vitro but it also makes a very important point, consistent with and extending the observations shown in Figure 1 and 3. Panel A shows that immature and mature DC are equally transfectable by mRNA via electroporation, exppressing high levels of GFP. Yet, as shown in Figure B, immature DC stimulate a more potent CTL response. In fact, immature DC transfected with mRNA and then matured in vitro are the best

stimulators of CTL. This unexpected, and counterintuitive, observation is consistent with the view that immature DC are effective at processing antigen whichis downregulated in mature DC whereas mature DC are better antigen presenting cells. Regardless, this is an important observation as it provides a rationale for how to load DC with RNA encoded antigens.

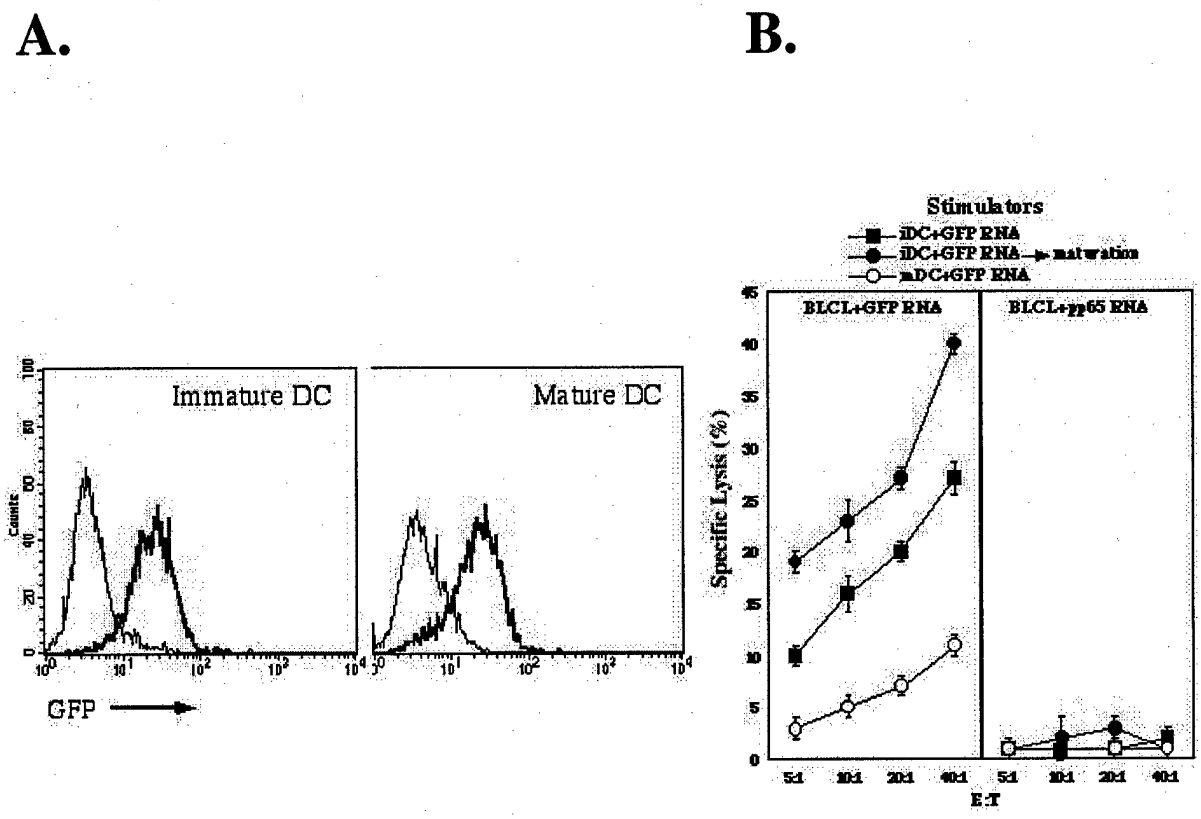

Figure 8: Electroporation of GFP mRNA into human DC-importance of state of maturation. Immature and cytokine cocktail matured DC were generated from monocytes and electroporated with GFP mRNA. Panel A: Expression of GFP measured by flow cytometry. Panel B: Stimulation of GFP specifi CTL from the PBMC of a healthy volunteer. BLCL electroporated with GFP mRNA were used as targets.

1-4. Generation of nuclease resistant "RNA".(SOW Year 2)

As described in the original application, we wanted to increase the nuclease resistance of the in vitro generated (amplified) RNA by incorporating modified pyrimidine during the transcription reaction. We chose to make modified GFP RNA and look at protein expression by flow cytometry and compare the results with non-modified GFP RNA. *In vitro* transcription was performed with pyrimidines that had either amino- or fluoro- substitutions at the 2' positions. In addition we tried to incorporate deoxy-pyrimidines into the reaction. Transcription reactions were carried out using either the MEGAscript kits (Ambion) or R&DNA Polymerase (Epicentre) which is a mutant RNA polymerase that allows higher levels of incorporation of modified nucleotides. We found that the R&DNA polymerase combined with the buffer from the MEGAscript kits gave the best yields of RNA, however, this was still less than we got with the unmodified nucleotides and normal polymerase. By pooling several reactions together we were able to get enough modified RNA to attempt transfection into F10.9 cells using the liposomal reagent DOSPER (Boehringer-Mannheim). Unfortunately, to date we were unable to detect GFP expression from the modified transcripts.

2. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with RNA isolated directly from tumor cells (Specific Aim #2). (SOW Year 2)

We have initially evaluated 35 patients (34 women, 31 Caucasion, 2 African American, and 1 male caucasion) with breast cancer for isolation of breast cancers cells for preparation of total tumor RNA.

Due to the use of stem cell growth factors, and the ability to harvest progenitor cells from mobilized blood, we have noted that bone marrow aspiration to harvest bone marrow for autologous bone marrow transplant is done much less frequently than previously. We attempted to utilize an alternative source of tumor cells, specifically, isolated tumor cells in other sites such as malignant ascites or malignant pleural effusions.

Twenty seven patients were not good candidates for isolation of purified breast cancer cells because they lacked pleural effusions or ascites which were accessible for drainage to obtain tumor cells. Five patients with metastatic breast cancer underwent therapeutic thoracentesis (three on two occasions for a total of 8 thoracenteses). All the thoracentesis specimens were processed with attempts to isolate sufficient quantities of tumor cells to generate total tumor RNA. In none of the cases were there enough tumor cells to isolate total tumor RNA. Therefore, no patient's breast cancer has been utilized to generate total tumor RNA that can be utilized in a clinical trial to date.

We have therefore begun to develop an alternative strategy for obtaining breast tumor cells using immumoisolation columns to isolate breast tumor cells from either bone marrow, or mobilized peripheral blood monuclear cells using antibodies and maganetic beads to "purge" the blood or bone marrow of tumor cells, generating a tumor cell preparation. This work is done in collaboration with investigators at Nexell Therapeutics, who have the antibodies, magentic beads, and columns necessary to "purge" bone marrow and mobilized peripheral blood monuclear cells. Pilot studies performed by collaborator Dr. Amy Ross at Nexell showed that the tumor enrichment columns (TECs) were capable of recovering from 15% to 67% of tumour cells in PBMC and bone marrow samples, for an enrichment that is 20-2500 fold. Protocols for isolating breast tumor cells were developed but despite intensive efforts, invariably generated insufficient numbers of tumor cells for RNA isolation and vaccination.

Significant delays in accruing patients was caused by the fact that Duke University had to undergo an extensive review for possible violations in the institutional IRB policy and IRB documentation. During this review, the clinical research activities of Duke investigators were suspended. In addition, the Duke IRB requested an audit and re-review of the hundreds to thousands of open protocols at Duke. This created a tremendous backlog of administrative work, and a backlog of protocols awaited review. Our protocol was reviewed and ultimately approved. Due to this review, we could not enroll patients for about 6 months.

As will be discussed in section 4 below, due to the regularotyr and technical difficulties in isolating sufficient amounts of tumor tissue for RNA isolation, and the rapid progress we made in developing RNA amplification protocols (Section 3), the attempts to initiate clinical studies with directly isolated tumor RNA were abandoned in favor of using amplified RNA (Specific Aim #4).

3. To develop methods to isolate, amplify, and enrich for biologically active mRNA from breast cancer tissue (Specific Aim #3).

The source of tumor tissue used to generate cDNA libraries was CEA positive tumor biopsies provided as frozen sections.

3-1. Proof-of-concept studies: mircodissection of tumor tissue, RNA amplification and CTL induction.

Microdissection: (SOW year 1 & 2) We used initially the SURF procedure (11) described in the application but were generally unsuccessful in adapting this protocol to our samples. One reason may be that it was developed for paraffin-embedded tissue and we are using frozen section (in order to preserve the intactness of the RNA). We have therefore adopted a simpler, though more labour intensive two-step procedure involving microscope guided scraping of normal material sorrounding the tumor nodule followed by collection of the tumor material. Figure 9A shows an H&E stained frozen section from a CEA^+ colorectal liver metastasis showing a metastatic nodule surrounded by benign tissue. To isolate tumor cells, normal tissue surrounding the cancerous nodule is first removed (Figure 9B) and then the tumor cells are collected (Figure 9C). In this experiment, 35 nodules were isolated in a 3 hr procedure from two consecutive sections, pooled, and RNA was extracted. RNA yield was 2.24 μg, obtained from approximately 55,000 tumor cells. Gel analysis under denaturing conditions and ethidium bromide staining confirmed that the RNA was intact (data not shown). As pointed out, the procedure is labor intensive requires exquisite skills and not always reproducible. We have begun to explore the use of laser guided microdissection (LTM) protocols.

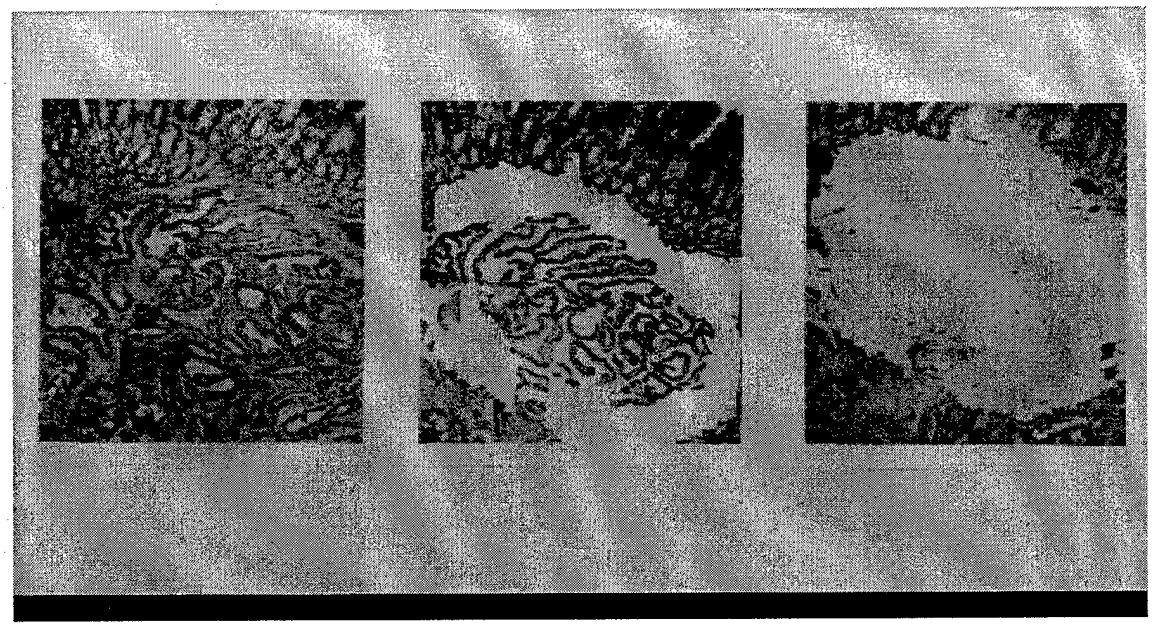

Figure i: Microdissection of a colorectal metastatic nodule from a frozen section. A liver metastasis was resected for curative intent from a patient with CEA positive colorectal cancer. Frozen sections were cut at 20 nm in a cryostat mounted on glass slides and stained with H&E. Panel A shows a single tumor nodule sorrounded by normal tissue. Microdissection was performed using an inverted microscope and an attached mechanical micromanipulator for manipulating a glass capillary tube which is used as a cutting tool. Under 40X-400X magnification, tumor cells are separated from benign tissue by first removing the normal cells adjacent to the tumor nodule (B) and then collecting the tumor tissue in the middle (C). 35 tumor nodules were isolated and pooled from 2 slides and RNA extracted using phenol/chloroform and ethanol precipitation. RNA yield determined by UV absorption was 2.24 μg. The integrity of the RNA was confirmed by agarose gel electrophoresis under denaturing conditions and ethidium bromide staining (data not shown).

cDNA library generation: (SOW Year 1) We have adopted and optmized the SMART PCR cDNA Synthesis protocol from Clonotech combined with the RNase H⁻ MMLV RT from Gibco/BRL, as described in the original application. The cDNA preparations are used to transcribe RNA in vitro with T7 polymerase (the T7 promoter is encoded in the amplification primers) and the products are first analyzed on denaturing agarose gels, first by staining with ethidium bromide and subsequently blotted and hybridized to an actin probe. An example is shown in Figure 10.

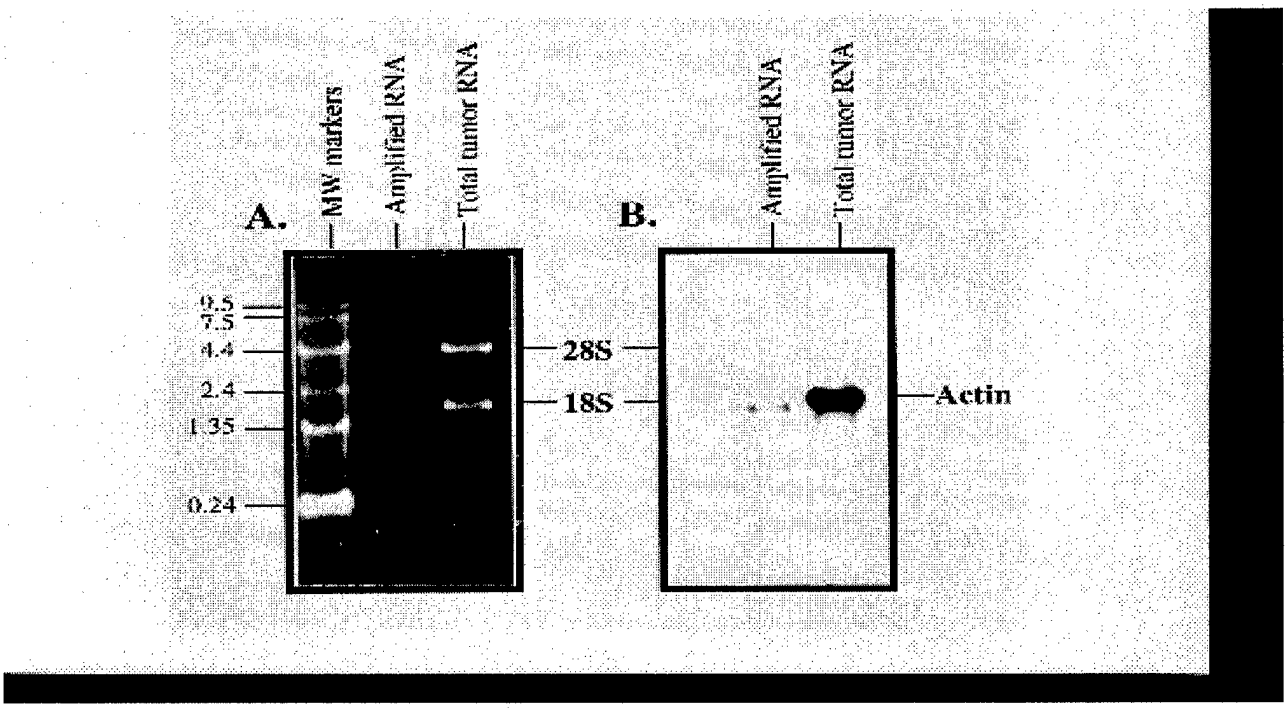

Figure 10: Biochemical analysis of RNA amplified from tumor cells. Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) following the manufacturers protocol. 1μg of RNA was amplified using the Smart PCR synthesis protocol (Clonotech). Tumor RNA and the amplified RNA products were subjected to agarose/formaldehyde gel electrophoresis and stained with ethidium bromide (A). Alternatively, RNA was blotted and hybridized with an actin probe (B).

Staining with ethidium bromide (Figure 10A) shows that the predominant RNA species present before amplification correspond to the two ribosomal RNA species while the amplified RNA migrates as a heterogeneous population corresponding in size to the mRNA population in murine cells. To assess whether the amplified RNA species correspond to full length transcripts, the RNA displayed in Figure10A was blotted and hybridized with an actin-specific random primed probe. In each case a single prominent band was seen which corresponds in size to the actin mRNA (Figure 10B).

Judging from the band intensities we estimate that the efficiency of generating full length actin during the amplification procedure was not more than 1-3%. These data show that the average size of the amplified RNA product corresponds to the expected size of the starting mRNA population, however, the efficiency of genrating full length RNA appears to be low; clearly there is room for improvement. We are currently testing various parameters of the amplification protocol. Next year we will start using alternative amplification strategies such as using oligo(dT) primers linked to paramagnetic beads for mRNA capture and "antisense RNA" techniques described in the original applications.

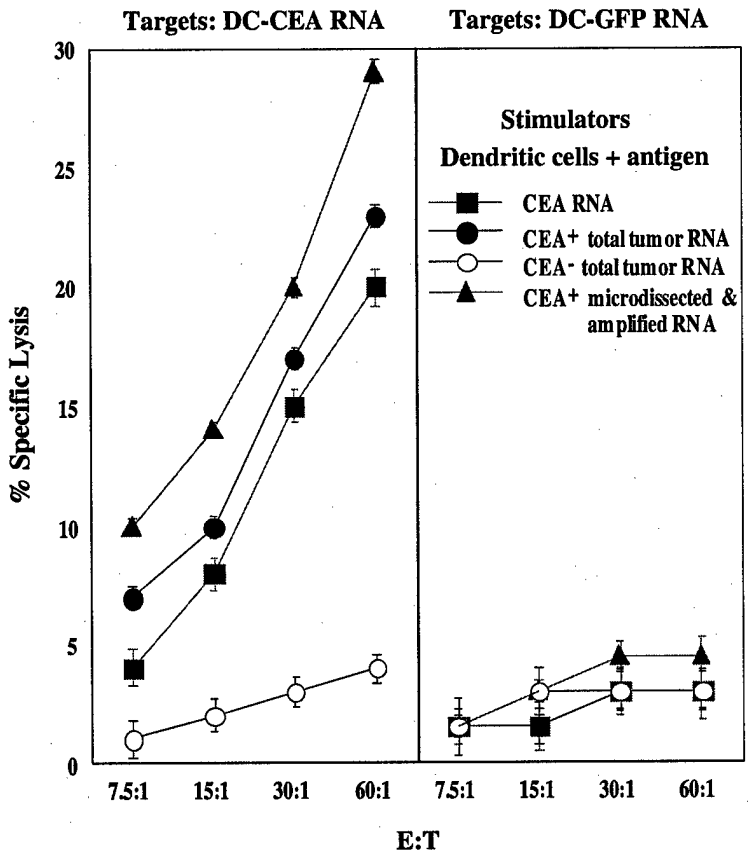

Figure 11: Induction of a primary, CEA-specific CTL response in vitro using DC transfected with RNA amplified from microdissected tumor cells. PBMC from a healthy volunteer were stimulated with autologous DC transfected with various RNA preparations and tested for the presence of CEA-specific CTL. DC transfected with in vitro synthesized CEA RNA or GFP RNA were used as specific and non specific targets, respectively, as previously. For stimulations, DC were transfected with in vitro transcribed CEA RNA, RNA isolated from a CEA^+ cell line (SW1463), a CEA^- cell line(KLEB), and RNA amplified from the microdissected tumor cells described in Figure 9.

Functional analysis of amplified RNA: The biological activity of the amplified RNA was demonstrated in a CTL assay shown in Figure 11. Clearly, amplified RNA transfected DC stimulate a robust CTL response despite the fact that by gel analysis only a fraction of the mRNAs are full length and hence translatable (see more examples below). RNA amplification was successfully extended to prostate cancer (not funded by this grant) (12).

3-2. Optimization of RNA isolation and amplification from microdissected tumor tissue. (SOW Year 3)

As described above, wewere able to generate cDNA libraries as well as microdissect and amplify the cDNA and recover the "amplified" RNA template. However, the protocols was considerably suboptimal and we readily identified one major problem. The existing amplification protocol employed a cap switch oligo to ensure the generation of full-length cDNAs and to provide a 5' primer sequence for amplification. As shown in Figure 12, in the current protocol a significant portion of spurious DNA is generated, most likely due to internal priming by the cap switch oligo on the RNA template. We have designed a new cap switch oligo with a blocked 3' end which has apparently solved this problem.

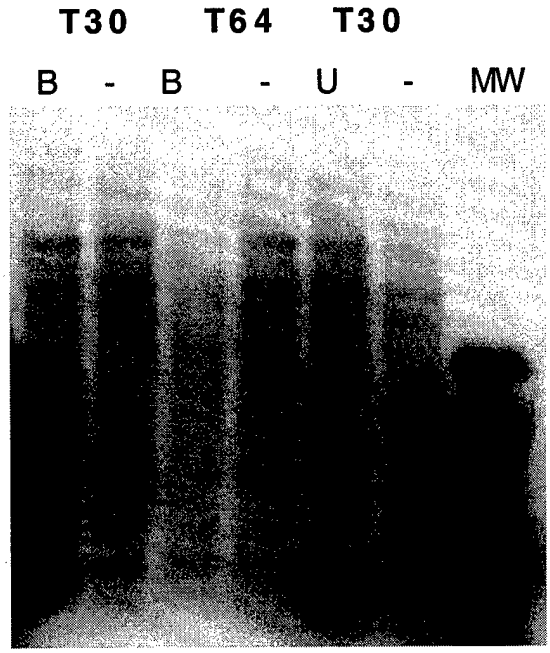

Figure 12: Evidence for internal priming by the cap switch oligo. SW403 RNA (SW403 is a colorectal tumor cell line) was placed in an RT reaction with 32P-dCTP: and the indicated RT primers (T30-30 Ts; T64-64 Ts) and either the unblocked (U), blocked (B) or no (-) cap switch oligo. The presence of additional bands in the presence of unblocked, but not blocked cap switch oligo are clear evidence of internal priming by the unblocked oligo.

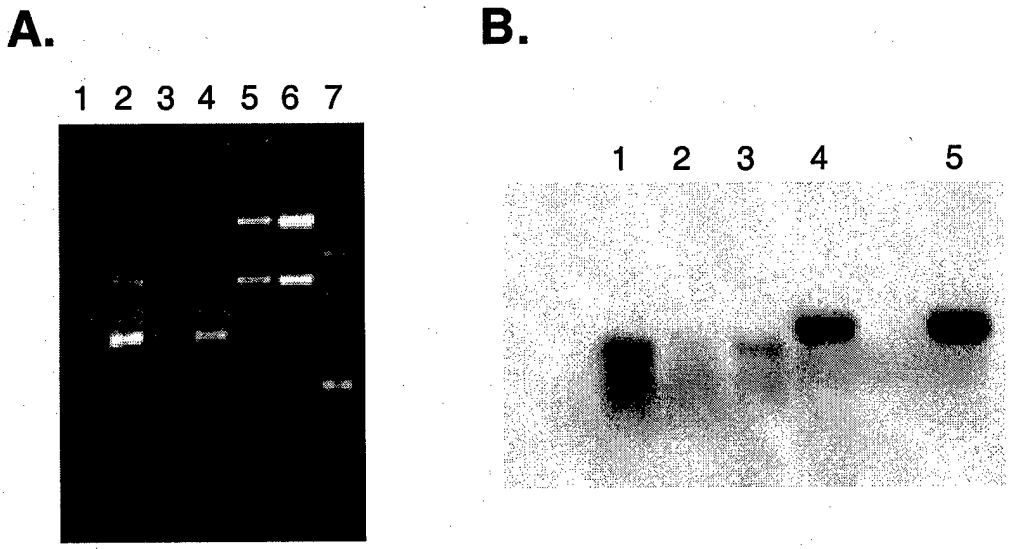

Figure 13: Amplification of RNA from tumor cells isolated from a breast cancer patient (BL). A. Ethidium bromide staining. **B.** Blotting with an actin probe. Lanes1and 2-using the T30 RT primer and blocked (lane 1) or unblocked (lane 2) cap switch oligo. Lanes 3 and 4 using the T64 RT primer and blocked (lane3) and unblocked cap switch primer (lane 4). Lanes 5 and 6, total RNA from patient BL and colorectal cell line SW403, respectively. Lane 7, MW markers.

Figure 13 shows the result of an amplification reaction using tumor RNA isolated from a breast cancer patient using either the blocked or unblocked oligo.

We are also developed improved methods to directly amplify RNA from microscopic amounts of tumor tissue circumventing the need to first isolate RNA. Two methods, using either heating in the presence of RNase inhibitors or freeze-thawing, are illustrated in Figure 14.

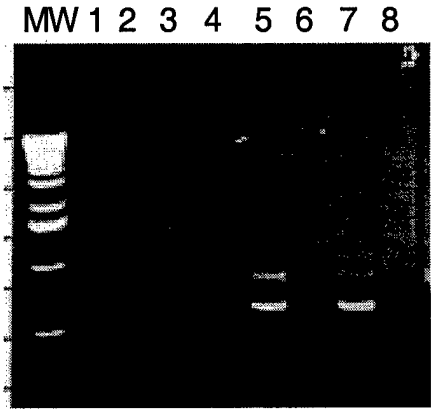

Figure 14: Direct amplification of RNA from tumor cells. 2000 F10.9-3.1 or F10.9-OVA cells, were lysed by heating to 75 oC in the presence of SUPERasin (Ambion), a broad spectrum heat-resistant RNase inhibitor, or by freeze-thawing in the presence of placental ribonuclease inhibitor. 250 cell equivalents were put into an RT reaction +/- the addition of reverse transcriptase. The cDNA was amplified for 30 cycles and 1/10 of the PCR products were analyzed. Lanes 1-MW markers; lanes 2 to 5-SUPERasin protocol; lanes 6 to 9 freeze-thaw protocol; lanes 2,3,6,7-F10.9-3.1 cells; lanes 4,5,8,9-F10.9-OVA cells; lanes 2,4,6,8-with RT; lanes 3,5,7,9-without RT..

3-3. Reproducibility of the mRNA amplification protocol

For clinical applications it was essential to demonstrate that the tumor mRNA amplification protocol is reproducible from a quantitative standpoint and biological activity.

Figures 14 and 15 shows the analysis of 5 separate samples of amplified RNA obtained from breast cancer tissue. Figure 14 shows that the amount of in vitro transcribed RNA produced was similar in each of the amplifications (range 25-28mcg), despite the fact that there was more variability in the amount of cDNA template produced (range 3.8-5.1 mcg). All three mRNAs evaluated (Actin, GAPDH, and CEA) were amplified in the final product of each separate amplification. We believe that the difference in lengths of the mRNA between the total tumor RNA and the amplified-RNA reflect the length of the polyA tails. Native mRNA generally has a PolyA tail of 100-200 bases, but our in vitro transcribed mRNA has 64 bases. Figure 15 shows that three amplified RNA samples tested stimulated similar levels of CTL responses.

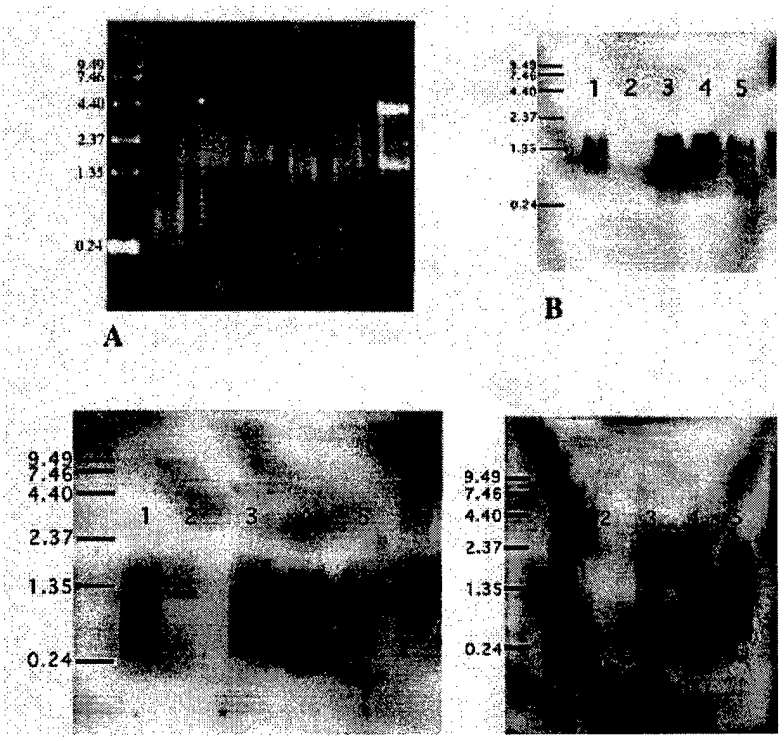

Figure 14: Reproducibility of amplifying RNA from tumor tissue. Total RNA was isolated from 0.9 g of breast tumor tissue using a Qiagen RNeasy Maxi kit and following the manufacturer's protocol. Total RNA (1 μg per reaction) was used in the standard amplification reaction. A total of five reactions were performed on three different days. RNA was produced *in vitro* from 1 μg of each of the amplified cDNAs. The *in vitro* transcribed RNA and total RNA (2 μg of each) was electrophoresed on a formaldehyde/ agarose gel and blotted to nylon membranes. Blots were probed separately with random-primed probes, which hybridize to actin, GAPDH and CEA messages.

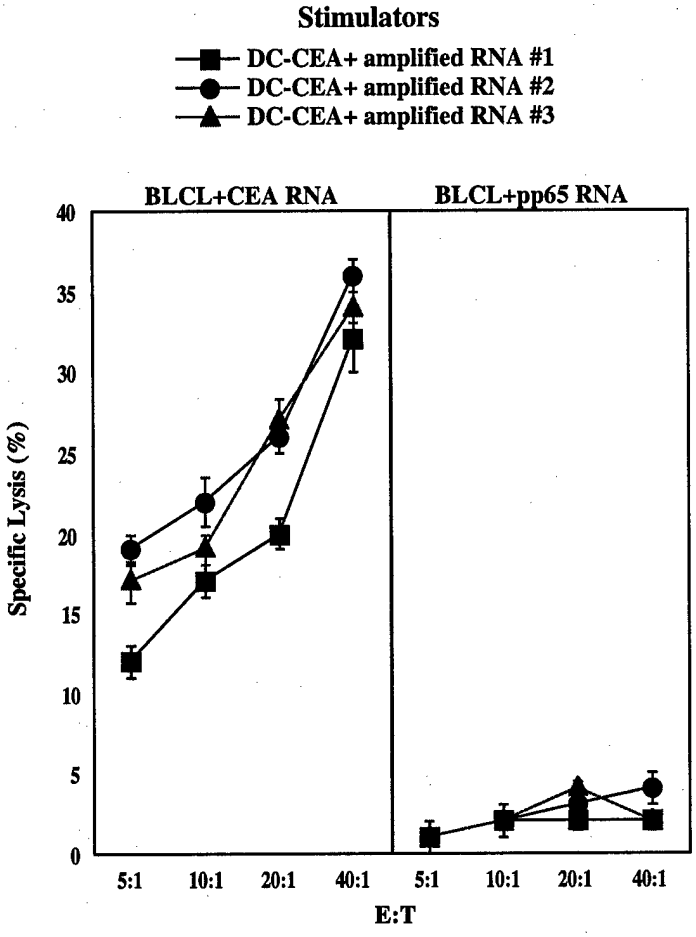

Figure 15. Reproducibility of CTL priming with amplified RNA from breast tumor tissues. RNA was isolated from a tumor specimen obtained from a patient with breast cancer, divided into three aliquots and amplified. Amplified RNA was transfected into H-2 matched DC and incubated with PBMC. After two cycles of stimulation, the generation of CEA-specific CTL were determined using a standard cytotoxicity assay using H-2 BLCL (autologous to the DC and PBMC) transfected with CEA RNA and control (CMV pp65) RNA.

4. To perform a phase I clinical trial of active immunotherapy in patients with refractory or recurrent metastatic breast cancer using autologous DC transfected with <u>amplified</u> tumor RNA. (SOW Year 4)

As described in section 1 and 3 (Specific Aims #1 and #3) we developed a robust protocol for isolating and amplifying tumor RNA from small amounts of tumor tissue and efficiently load human DC with the amplified RNA-as evidenced by the ability to stimulate a potent CTL response in vitro. As also mentioned above, the plans for conducting a trial with non amplified tumor RNA was defeated due to technical and regulatory difficulties. The clinical strategy was therefore altered to use a method that would not rely on a minimal volume of cells for the generation of total tumor mRNA, specifically, the amplification of total tumor mRNA by reverse transcriptase polymerasae chain reaction (RT-PCR) into a clinically testable vaccine strategy. This required, in addition to the results of the earlier aims, applications to regulatory authorities (in particular, the FDA) to use the optimal strategy in humans. We prepared an amendment to the clinical protocol and to the FDA IND to use total tumor mRNA amplified by RT-PCR to generate sufficient mRNA to pulse onto autologous dendritic cells.

On July 21, 2000, we arranged a teleconference with the FDA clinical, product, and toxicology reviewers. We initiated discussion regarding the requirements for validating the approach of RT-PCR amplification of total tumor RNA for use in dendritic cell-based immunizations during the teleconference. Based on comments and queries from the FDA clinical, product, and toxicology reviewers, further preclinical testing was required prior to initiating clinical trials with RT-PCR amplified total tumor mRNA. We then prepared an amendment to the clinical protocol and to the FDA IND that was responsive to the concerns raised.

On January 18, 2001, we submitted to the FDA clinical, product, and toxicology reviewers an amendment to our existing IND (#6888) in which we proposed the phase I clinical trial with RT-PCR amplified total tumor RNA. We sent supporting data generated in the Gilboa laboratory which consisted of Northern Blot data from tumor RNA showing that the tumor RNA could be amplified and we also supplied data showing that cytolytic T cells specific for the tumor antigen CEA could be induced by dendritic cells loaded with tumor RNA (containing CEA mRNA) following amplification.

On March 7, 2001, the FDA clinical, product, and toxicology reviewers requested additional data. In particular, the FDA clinical, product, and toxicology reviewers requested that we provide data to show that CTL can be induced using amplified RNA obtained from at least three fresh tumor samples compared to total RNA from the same tumor samples. We again prepared an amendment to the clinical protocol and to the FDA IND that was responsive to the concerns raised.

On April 24, 2001, we communicated to the FDA clinical, product, and toxicology reviewers that we did not think it was feasible to obtain large enough tumor specimens from breast cancer patients to extract adequate RNA to compare amplified and non-amplified RNA-loaded DC. We supplied additional data from Dr. Gilboa's laboratory showing that human DC loaded with microdissected, amplified RNA from a CEA expressing tumor stimulated CTLs with greater activity against a CEA-expressing target compared with total tumor RNA from the same CEA-expressing tumor.

On May 2, 2001, we then held a follow-up telephone conference with the FDA clinical, product, and toxicology reviewers during which we were requested to perform the same type of experiment that yielded this additional data, at least 2 more times. We attempted to prepare an amendment to the clinical protocol and to the FDA IND that was response to the concerns raised but were unable to generate these data due to the unavailability of clinical breast cancer specimens. We arranged another teleconference with the FDA to address these technical limitations.

On June 19, 2001 we held another teleconference which was held on June 19, 2001 at which time the FDA clinical, product, and toxicology reviewers requested that Dr. Gilboa's group use mRNA from tumor cell lines and not fresh tumor to obtain the needed data.

On Augurst 29, 2001, the Duke University Medical Center IRB re-approved the clinical trial protocol, but could still not enroll patients because of the outstanding issue with the FDA clinical, product, and toxicology reviewers regarding reproducibility of tumor RNA amplification. We prepared an amendment to the clinical protocol and to the FDA IND that was responsive to the concerns raised.

On December 28, 2001 we provided data to the FDA clinical, product, and toxicology reviewers demonstrating that when mRNA from a CEA+ breast cancer specimen is amplified (3 separate times), the mRNAs for actin and the tumor antigen CEA were reproducibly amplified and present in relatively similar amounts in the final product. Furthermore, dendritic cells loaded with this amplified mRNA could stimulate similar levels of CEA-specific cytolytic activity. We did receive verbal confirmation in the late spring of 2002 from the FDA clinical, product, and toxicology reviewers that they had received

and reviewed the supplemental data and were in agreement with us proceeding to the clinical trial. (Although we have not received a written response from the FDA)

Since the last communication with the FDA clinical, product, and toxicology reviewers, we activated the study, and have been actively screening patients with metastatic breast cancer for enrollment, but have not identified a patient with a metastatic CEA-expressing breast cancer for whom tumor tissue was available in a form that would permit us to perform mRNA amplification for use in generating a dendritic cell vaccine.

One of the complicating factors has been in the use of the antibody Herceptin in many trials in combination with chemotherapy in patients with metastatic breast cancer. In addition, a large source of patients at Duke, those undergoing autologous bone marrow transplant and high dose chemotherapy, was eliminated based on large clinical trials results reported by the clinical oncology societies, suggesting that there was no survival benefit from receiving high dose chemotherapy. For examples, from a clinical volume of over 300 patients per year undergoing high dose chemotherapy and bone marrow transplant for stage IV breast cancer, the Duke University Bone Marrow Transplant Program currently expects about 50-60 patients per year. Finally, tissue banking became a significant issue for breast cancer patients at Duke with the implementation of an for-profit tissue banking company, Ardias, that has earnestly begun to acquire tissue for scientific study by academic and commercial investigators. Therefore, no patients have been enrolled in the therapeutic phase of the trial. Since the funding period for this grant is completed, we do not plan to continue screening patients for this study. We will not renew the IRB approved protocol for this study.

C. KEY RESEARCH ACCOMPLISHMENTS

1. Development of an exceptionally robust and efficient method to load human dendritic cells with antigens-maturing monocyted derived DC with cytokine cocktail and electroporated with mRNA encoding tumor antigens and CD40 ligand.

2. Development of a new approach to modulate the bioactivity of cells ex vivo by transfecting cells with mRNA encoding biological response modifiers.

3. Development of efficient and reproducible mRNA amplification methods from breast cancer tissue to provide an inexhaustible amount of antigen for vaccination protocols.

4. Approval of INDs for clinical trials in breast cancer patients using amplified tumor RNA transfected autologous DC .

D. REPORTABLE OUTCOMES

1. Modulation of human dendritic cell generation and maturation by transfection with mRNA encoding IL-4 and CD40 ligand (manuscript in preparation).

E. CONCLUSIONS

1. The studies funded by this grant applications have accomplished the stated preclinical goals: a) a robust DC antigen loading was achieved incorporating two novel elements-not anticipated when application was submitted-transfection of mRNA by electroporation and maturation of DC

transfection with CD40 ligand mRNA. b) We were successful in developing methods to amplify mRNA from breast cancer tissue. The procedure prooved to be reproducible and yielded biologically active RNA, namely RNA that upon transfection of DC was able to stimulate a CTL response.

These studies, while focusing on breast cancer, are of general value for other forms of cancer. In fact, preclinical and clinical studies incoporating various elements of this research are currently in progress in our institution in glioma, prostate cancer and renal cancer.

2. The clinical applications of this strategy were stymied by difficulties of obtaining sufficient tumor tissue for antigen preparation (Aim #2) and later on, accrual of patients (Aim #4) as discussed in the body section. The difficulties in accrual, precipitated by the success of herceptin therapy suggest that breast cancer may not be the appropriate target for developing novel and yet untested vaccination strategies. This however remains to be seen.

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G. APPENDICES –N/A